Optimization of the annealing temperature for DNA amplification *in vitro*

W.Rychlik*, W.J.Spencer[1] and R.E.Rhoads

Department of Biochemistry, Medical Sciences Building, University of Kentucky, Lexington, KY 40536 and [1]The Perkin-Elmer Corporation, Northpointe Business Park, Fairfield, OH 45014, USA

ABSTRACT

In the polymerase chain reaction (PCR) technique, DNA is amplified *in vitro* by a series of polymerization cycles consisting of three temperature-dependent steps: DNA denaturation, primer-template annealing, and DNA synthesis by a thermostable DNA polymerase. The purity and yield of the reaction products depend on several parameters, one of which is the annealing temperature (T_a). At both sub- and super-optimal T_a values, non-specific products may be formed, and the yield of products is reduced. Optimizing the T_a is especially critical when long products are synthesized or when total genomic DNA is the substrate for PCR. In this article we experimentally determine the optimal annealing temperature (T_a^{OPT}) values for several primer-template pairs and develop a method for its calculation. The T_a^{OPT} is found to be a function of the melting temperatures of the less stable primer-template pair and of the product. The fact that experimental and calculated T_a^{OPT} values agree to within 0.7°C eliminates the need for determining T_a^{OPT} experimentally. Synthesis of DNA fragments shorter than 1 kb is more efficient if a variable T_a is used, such that the T_a is higher in each consecutive cycle.

INTRODUCTION

One of the most powerful techniques currently used in molecular biology is DNA amplification by polymerase chain reaction (PCR) using a thermostable DNA polymerase isolated from *Thermus aquaticus* (1). Under optimal conditions the reaction is very efficient; microgram quantities may by synthesized from a single molecule of substrate DNA. Ordinarily, several experiments are required to achieve optimal conditions for PCR, even if good primers are chosen, i.e., those that do not form dimers by annealing of their 3'-termini and that are specific to one sequence in the substrate DNA. Optimization of PCR involves testing a number of variables, one of which is the annealing temperature (T_a). If the T_a is too low, non-specific DNA fragments are amplified, causing the appearance of multiple bands on agarose gels. If the T_a is too high, the yield of the desired product, and sometimes the purity is reduced due to poor

annealing of primers. In this work we have examined the T_a value at which maximal yield of the desired product is obtained (T_a^{OPT}). A method for T_a^{OPT} determination is presented which is based on the calculated melting temperatures (T_m) of the less stable primer-template pair and of the product.

METHODS

Calculations of T_m were performed using the computer program OLIGO (2). The standard PCR conditions were: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 μM of each primer, 0.2 mM of each dNTP, including [α-^{32}P]dATP, 0.1 ng of eIF-4E cDNA plasmid (3) and 2.5 U to *Taq* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, PA) in a 100 μl reaction mixture. Each step was for 1 min unless otherwise noted: denaturation at 94°, annealing at temperatures indicated, and polymerization at 72°. Reactions were incubated in a Perkin-Elmer/Cetus Thermal Cycler at six temperature points per experiment (at 1−5° intervals). The amount of synthesized DNA was determined by one of two methods. In one, the purity of the product was checked by agarose gel electrophoresis and, in most cases, the whole reaction mixture was precipitated and subsequently washed with 10% trichloroacetic acid. In some cases the specific product was excised from the gel and counted. The other method was by ethidium bromide staining followed by densitometric scanning of the negative. For this method, DNA was compared to a standard DNA consisting of a known amount (determined spectrophotometrically) of the 448-bp PCR product described below, which was purified by adsorption on glass particles (GeneClean kit, RPI, La Jolla, CA). In either case, the T_a^{OPT} was determined from a third order polynomial fit to the six temperature data points in a given experiment, using SigmaPlot software (Jandel Scientific, Corte Maders, CA).

RESULTS AND DISCUSSION

Eleven pairs of oligodeoxynucleotide primers complementary to either bacteriophage λ DNA or protein synthesis initiation factor 4E (eIF-4E) cDNA (3) were used to establish empirically a range of T_a^{OPT} values. The primers were selected to be highly specific and not form dimers longer than 3 bp at their 3'-termini (Table

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* To whom correspondence should be addressed

Table I. Primers used in PCR experiments

DNA Source, Strand Sense	5'-end Position Number	Primer Length (nt)	Primer Sequence (5' to 3')	Calcu-lated T_m^1 (°C)	Longest 3'-end Overlap (bp)
eIF-4E +	10	21	GTCGAACCGGAAACCACCCCT	60.7	1
eIF-4E −	218	23	GTGGATATGGTTGTACAGAGCCC	54.3	3
eIF-4E +	323	18	AACGGGGAGGACGATGGC	56.8	2
eIF-4E −	440	18	CAGCGCCACATACATCAT	46.9	1
eIF-4E −	695	22	TCAGCTCCCAAATCTCGATTGC	58.0	2
eIF-4E +	885	24	TACACATCCCCAGAATCCATAAAT	53.4	2
eIF-4E +	1287	18	CAAAGGTGGAATAAACAT	39.0	2
eIF-4E −	1520	24	TAACCAAAGCAAAATAACCTAAGT	48.9	2
lambda +	7131	25	GATGAGTTCGTGTCCGTACAACTGG	58.6	2
lambda −	7606	25	GGTTATCGAAATCAGCCACAGCGCC	65.2	2
lambda −	20161	25	CCGGCGCAGAAGCGGCATCAGCAAA	73.9	1
lambda −	24182	25	TCACCAGCCAGCCGCAGCACGTTCC	72.8	1
lambda +	30042	25	AGGATGGCGAACAACAAGAAACTGG	61.3	1
lambda −	31017	25	GGCGAAAGCAGAAGCAGATGAGAGA	61.9	1

[1]Calculated from Eqn. (ii) using c = 250 pM and K^+ = 50 mM.

I). For each primer pair, the T_a was varied and the yield of product determined. A typical experiment, in which a 135-bp product was synthesized from eIF-4E cDNA using primers 323 and 440, is shown in Fig. 1. At 25 cycles, determining the T_a^{OPT} was difficult because the reaction had essentially gone to completion and the yield of product was constant over a broad range of T_a values. At 17 cycles, on the other hand, the T_a^{OPT} was readily distinguishable. The T_a^{OPT} values for each of the primer combinations were determined experimentally and are presented in Table II. The two methods of DNA quantitation, scintillation counting and ethidium bromide staining, gave similar results.

In order to calculate T_a^{OPT}, we developed the empirical formulation:

$$T_a^{OPT} = 0.3T_m^{primer} + 0.7T_m^{product} - 14.9 \qquad (i)$$

in which T_m^{primer} is the calculated T_m of the less stable primer-template pair (Table I) and $T_m^{product}$ is the T_m of the PCR product.

To calculate T_m^{primer}, we turned to the nearest-neighbor model of Borer *et al.* (4) and the thermodynamic values of Breslauer *et al.* (5) to predict T_m values from nucleotide sequences. Since the latter were obtained in 1 M Na$^+$, it was necessary to add a term to correct for the fact that PCR reactions are conducted in 50 mM K$^+$ (the effect of K$^+$ and Na$^+$ on DNA hybridization temperature is very similar; 6), resulting in the equation:

$$T_m primer = \frac{\Delta H}{\Delta S + R \times \ln(c/4)} - 273.15 + 16.6 \ \log[K^+] \qquad (ii)$$

where ΔH and ΔS are the enthalpy and entropy for helix formation, respectively (5), and R is the molar gas constant (1.987 cal/°C×mol). In the formulation of Freier *et al.* (7), c is the total molar concentration of the annealing oligonucleotides when oligonucleotides are not self-complementary. Knowing what concentration to use for c in the case of a PCR experiment, however, is problematical: the concentration of template changes dramatically during the course of the PCR reaction (e.g., 260,000-fold in the experiment shown in Fig. 1, 25 cycles). We determined empirically that using c = 250 pM in Eqn. (ii) gave good agreement with experimental results for all primer-template combinations tested.
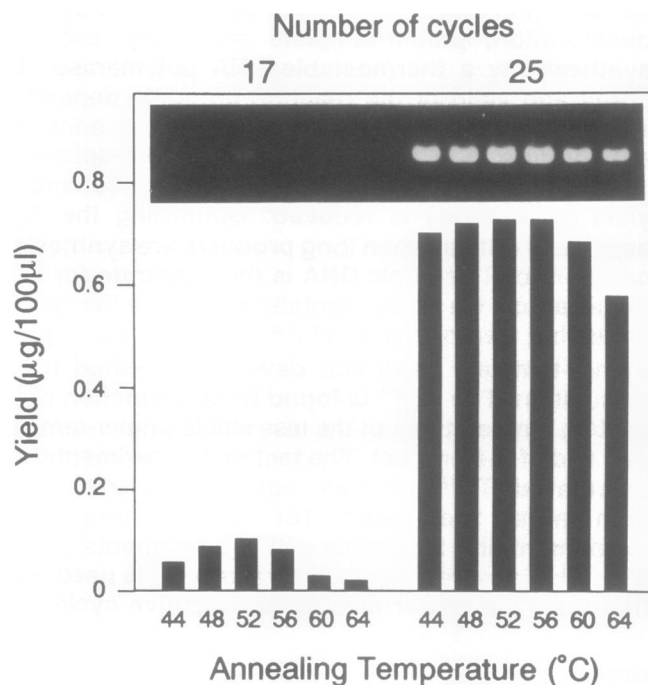

Figure 1. Synthesis of a 135-bp DNA fragment by PCR using eIF-4E cDNA and primers located at positions 323 and 440. The PCR was performed in the standard conditions as described in *Methods*. After 17 or 25 cycles, 5 µl of the DNA were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. *Upper panel*, the photograph of the gel; *lower panel*, quantitation based on densitometric scanning of the negative.

The second variable in Eqn. (i), $T_m^{product}$, cannot be calculated from Eqn. (ii) since the nearest-neighbor model is not applicable to long DNA molecules. Instead, the formula of Baldino *et al.* (8) with minor modifications was used:

$$T_m^{product} = 0.41(\%G + \%C) + 16.6 \ \log[K^+] - 675/l \qquad (iii)$$

where l is the length of the product in nucleotide residues. The K$^+$ concentration used in both Equations (ii) and (iii) was 50 mM.

Table II compares the T_a^{OPT} values for eleven primer-template

Table II. The optimal annealing temperature (T_a^{OPT}) of PCR

Pairs of Primers Used[1]	Product Length (bp)	Longest 3'-end hetero-duplex	T_a^{OPT} (°C)[2] Experimental			Calcu-lated	Yield[3] (μg/100 μl) T_a Used	
			mean	(n)	S.E.M.		Exp.	Var.
323−440	135	4	50.5	(5)	1.27	50.5	0.64	0.86
10−218	227	2	54.5	(4)	0.67	53.8	1.10	1.40
1287−1520	257	2	44.0	(3)	1.54	44.1	n.d.	n.d.
323−695	394	2	55.3	(6)	0.83	55.0	1.11	1.27
10−440	448	2	50.9	(7)	0.67	52.3	1.95	2.00
7131−7606	500	3	60.0	(4)	0.74	59.8	1.65	1.80
885−1520	659	2	48.4	(3)	0.55	49.2	1.70	1.78
10−695	707	2	56.0	(4)	0.38	55.8	1.67	1.77
30042−31017	1000	1	59.8	(4)	1.24	58.3	1.90	1.85
20161−24182	4046	2	59.8	(3)	1.71	61.4	1.95	0.62
20161−31017	10881	2	56.6	(5)	0.47	57.6	0.09	0.00

[1]Position numbers refer to the 5'-termini of oligonucleotides in Table I.
[2]T_a^{OPT} was either determined experimentally (17 cycles were performed) as described in the Fig. 2 legend or calculated from Eqn. (i); n, number of experiments; S.E.M., standard error of the mean.
[3]Using experimentally determined T_a^{OPT} (Exp.) or variable T_a (Var.); n.d., not determined. The mean values of 2−3 determinations per primer pair are given.

pairs determined experimentally and calculated from Eqn. (i). The average difference between experimental and calculated values was 0.7°. Table II also gives the yield of product for each primer-template pair at the experimentally determined T_a^{OPT}. Typically, the yield of the product was in the range of 1−2 μg/100 μl. Lower yields were observed with the primer pairs 323−440 and 20161−31017. In the first case this might be due to competition between primer-template and primer-dimer formation. This underscores the importance of selecting primers that do not form dimers or self-complementary duplexes at their 3'-termini for the best product yields. In the second case, where the DNA product was particularly long (10.9 kb), the low yield was most likely due to inhibition of the polymerase because of reannealing of the product before polymerization could occur.

Even though the T_a^{OPT} values were calculated on the basis of total yield of the desired product, we found that the purity of the product was also optimal at the T_a^{OPT}. Fig. 2 summarizes experiments in which the nature of product was examined. In the first series of reactions (A), a constant polymerization time of 20 min was used and the T_a varied. At temperatures both lower and higher than the calculated T_a^{OPT} (57°), non-specific products were formed. Those formed at lower temperatures were presumably due to annealing of primers to non-specific sites on the template. It is unclear why non-specific products were formed at temperatures higher than the T_a^{OPT}, but this was a consistent finding. At the T_a^{OPT}, the 10,881-bp product was not detected if the polymerization time was 10 min (Fig. 2B), despite published information which indicates that rate of DNA synthesis by the *Taq* polymerase is sufficient to complete a 10.9 kb product in this time (9). For a 30-min polymerization time, more product was obtained, but the product still consisted of essentially only one band. However, for this polymerization time, the range of annealing temperatures yielding the specific product was narrowed, resulting in a smear at both 54° and 61° but not at the T_a^{OPT} of 57° (Fig. 2C). In experiment not shown, where the standard PCR buffer was used, deviations of as little as 1° from the T_a^{OPT} produced similar results.

Fig. 3 shows the result of an experiment in which total human DNA instead of plasmid was used for PCR. It is worth noting that, at the predicted T_a^{OPT}, formation of non-specific product
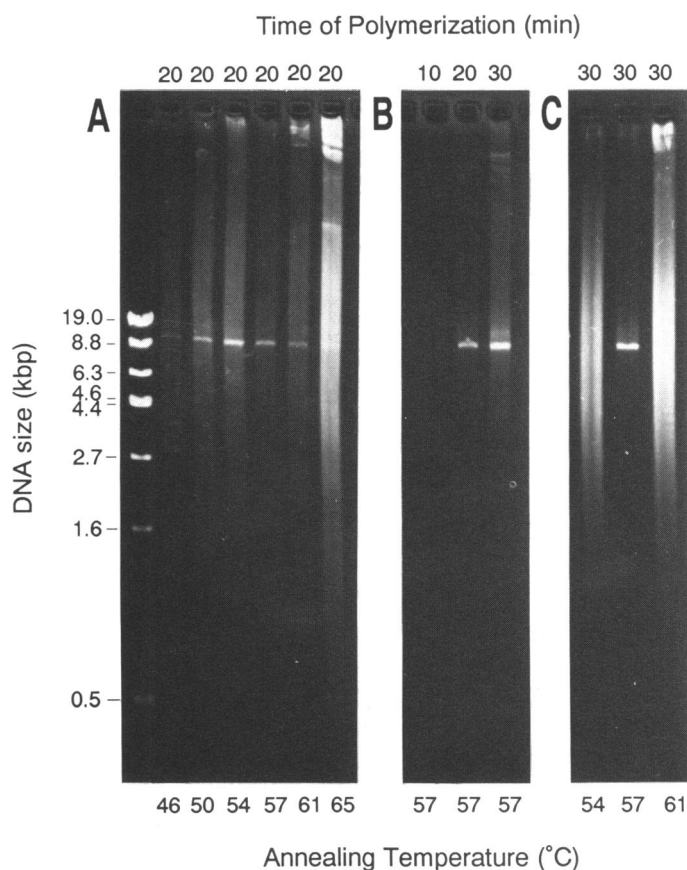

Figure 2. Synthesis of 10,881-bp DNA fragment from a bacteriophage λ template. The PCR was performed in the standard conditions except that 1.3 mM MgCl₂ and 1 ng of λ DNA were used and the polymerization temperature was 70°. Both the T_a and polymerization time were varied as indicated. After 32 cycles, 10 μl aliquots of the reaction mixture were analyzed. DNA size standards, *BclI*-treated bacteriophage λ DNA, are shown on the left.

was low. In all three reactions, the highest yields of the correct product were obtained at or near the calculated T_a^{OPT} (cf. Fig. 3 and Table II), despite the fact that much lower concentrations
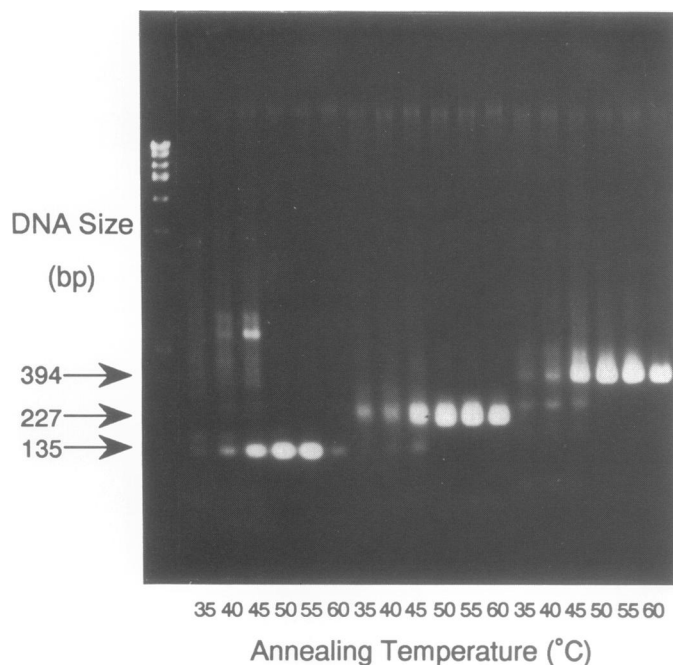
Figure 3. Synthesis of the 135-, 227- and 394-bp DNA fragments using total human DNA as template. Standard PCR conditions were followed except that 33 cycles and 0.5 μg of DNA were used. Eight microliter samples were analyzed. The DNA size standards were the same as in Fig. 2. Arrows indicate expected positions of specific products.
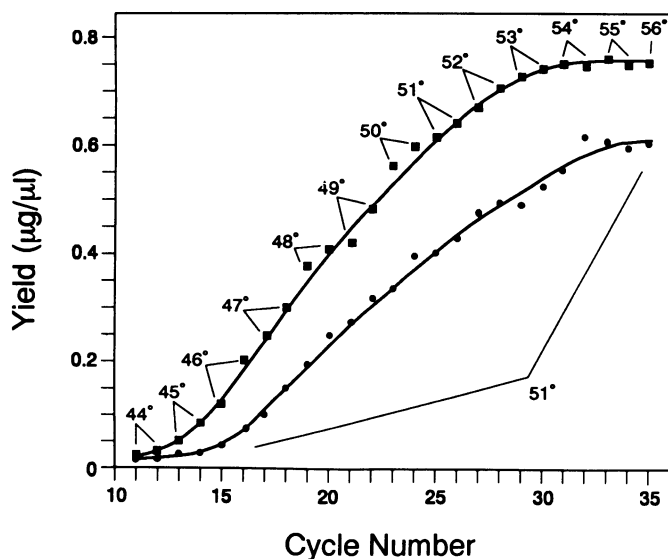

Figure 4. Comparison PCR using constant and variable T_a. Plasmid DNA was amplified using primers 323 and 440 as described in *Methods*. The T_a for each cycle is indicated. In the variable method, the initial T_a was 39° for the first two cycles, after which it was increased by 1° every other cycle.

of the template DNA were used compared to the T_a^{OPT} determination experiments. If the T_a was below optimum, non-specific bands were synthesized; if it was above optimum, the yield was decreased. We tested the dependence of initial template concentration on T_a^{OPT} explicitly by varying the starting amount of DNA. The T_a^{OPT} was the same between 1 pg and 10 ng of plasmid used (not shown).

As noted above, the concentration of the template changes dramatically throughout the course of a PCR experiment. One might expect that this would cause the T_a^{OPT} of each PCR cycle to be different. To test this, the T_a was varied throughout the course of a PCR experiment. For the first cycle, the T_a was determined by subtracting 8° from the T_m of the less stable oligonucleotide as calculated from the actual starting template concentration. The T_a was then increased by 1° every other cycle (Fig. 4). When a variable T_a was used, fewer cycles were required to obtain the same amount of product and, more importantly, a higher final DNA yield was observed. This procedure was applied to all template-primer pairs, and the yield of product is listed in Table II (column 'Var'). The best results for this new method were obtained with the 323−440 primer pair. The larger the product the less difference between the variable and constant T_a methods was seen. For products less than 1 kb, this method gave up to 33% better yield. Using a variable T_a for the synthesis of longer DNA products, however, reduced the yield (Table II), possibly due to poorer enzyme processivity (9) and less specific annealing of primers at the lower initial temperatures. The variable annealing temperature method using the total genomic DNA did not improve the yield (not shown). The yield increase was also observed when only the first few PCR cycles were performed at lower than T_a^{OPT} followed by several cycles at constant, optimal T_a (not shown).

In summary, it is possible to calculate the T_a^{OPT} for PCR based on published thermodynamic parameters and base composition. The calculated T_a^{OPT} values agree well with those determined experimentally. Use of the T_{aOPT} provides the highest absolute yield of the correct product and the fewest non-specific products. Choice of primers which do not significantly anneal with each other at either 3′-terminus improves the yield of PCR. For products less than 300 nt, using a variable T_a significantly increases the yield of product and reduces the number of cycles necessary to achieve maximal amplification.

ACKNOWLEDGEMENTS

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